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Influence of embryo culture medium (G5 and HTF) on pregnancy and perinatal outcome after IVF: a multicenter RCT

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STUDY QUESTION: Does embryo culture medium influence pregnancy and perinatal outcome in IVF?

SUMMARY ANSWER: Embryo culture media used in IVF affect treatment efficacy and the birthweight of newborns.

WHAT IS KNOWN ALREADY: A wide variety of culture media for human preimplantation embryos in IVF/ICSI treatments currently exists. It is unknown which medium is best in terms of clinical outcomes. Furthermore, it has been suggested that the culture medium used for the *in vitro* culture of embryos affects birthweight, but this has never been demonstrated by large randomized trials.

STUDY DESIGN, SIZE, DURATION: We conducted a multicenter, double-blind RCT comparing the use of HTF and G5 embryo culture media in IVF. Between July 2010 and May 2012, 836 couples (419 in the HTF group and 417 in the G5 group) were included. The allocated medium (1:1 allocation) was used in all treatment cycles a couple received within 1 year after randomization, including possible transfers with frozen–thawed embryos. The primary outcome was live birth rate.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Couples that were scheduled for an IVF or an ICSI treatment at one of the six participating centers in the Netherlands or their affiliated clinics.

MAIN RESULTS AND THE ROLE OF CHANCE: The live birth rate was higher, albeit nonsignificantly, in couples assigned to G5 than in couples assigned to HTF (44.1% (184/417) versus 37.9% (159/419); RR: 1.2; 95% confidence interval (CI): 0.99–1.37; $P = 0.08$). Number of utilizable embryos per cycle (2.8 ± 2.3 versus 2.3 ± 1.8 ; $P < 0.001$), implantation rate after fresh embryo transfer (20.2 versus 15.3%; $P < 0.001$) and clinical pregnancy rate (47.7 versus 40.1%; RR: 1.2; 95% CI: 1.02–1.39; $P = 0.03$) were significantly higher for couples assigned to G5 compared with those assigned to HTF. Of the 383 live born children in this trial, birthweight data from 380 children (300 singletons (G5: 163, HTF: 137) and 80 twin children (G5: 38, HTF: 42)) were retrieved. Birthweight was significantly lower in the G5 group compared with the HTF group, with a mean difference of 158 g (95% CI: 42–275 g; $P = 0.008$). More singletons were born preterm in the G5 group (8.6% (14/163) versus 2.2% (3/137), but singleton birthweight adjusted for gestational age and gender (z-score) was also lower in the G5 than in the HTF group (-0.13 ± 0.08 versus 0.17 ± 0.08 ; $P = 0.008$).

LIMITATIONS, REASONS FOR CAUTION: This study was powered to detect a 10% difference in live births while a smaller difference could still be clinically relevant. The effect of other culture media on perinatal outcome remains to be determined.

[†] These authors contributed equally to this article.

WIDER IMPLICATIONS OF THE FINDINGS: Embryo culture media used in IVF affect not only treatment efficacy but also perinatal outcome. This suggests that the millions of human embryos that are cultured *in vitro* each year are sensitive to their environment. These findings should lead to increased awareness, mechanistic studies and legislative adaptations to protect IVF offspring during the first few days of their existence.

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TRIAL REGISTRATION NUMBER: NTR1979 (Netherlands Trial Registry).

TRIAL REGISTRATION DATE: 1 September 2009.

DATE OF FIRST PATIENT'S ENROLMENT: 18 July 2010.

Key words: culture medium / IVF/ICSI / human preimplantation embryos / live birth / birthweight

Introduction

Subfertility is of major clinical and social concern. The most frequently used treatments of subfertility are IVF and ICSI. The American and European registries of assisted reproductive technology (ART) reported a total of 697 556 initiated ART cycles in the year 2010 (Centers for Disease Control and Prevention, 2012; Kupka et al., 2014). In the USA and Europe, a delivery rate per started cycle was reported of 29 and 24%, respectively (Centers for Disease Control and Prevention, 2012; Kupka et al., 2014).

The medium that is used for embryo culture in IVF/ICSI treatments is considered to play an important role in IVF/ICSI success rates and treatment outcomes. The choice of embryo culture medium has been found to affect embryo quality and pregnancy chances (Quinn et al., 1985; Utsunomiya et al., 2002; Balaban and Urman, 2005; Hoogendijk et al., 2007; Cossello et al., 2012; Mantikou et al., 2013). However, a recent systematic review showed that randomized studies that compare clinical outcomes of different culture media are limited in number and of low methodological quality (Mantikou et al., 2013; Youssef et al., 2015). Thus, it is yet unknown which culture medium leads to the highest live birth rates after IVF/ICSI, the outcome generally considered to be most relevant for evaluating IVF programs (Mantikou et al., 2013).

Children born after ART are at increased risk of adverse perinatal outcomes, such as preterm birth and low birthweight, when compared with naturally conceived children (Pandey et al., 2012; Fauser et al., 2014; Henningsen and Pinborg, 2014). This may be due to both intrinsic patient factors and specific aspects of the IVF technique (Pinborg et al., 2013).

Initial reports from a semi-randomized data analysis suggested that culture media in IVF could affect birthweight of the children (Dumoulin et al., 2010; Nelissen et al., 2012). The difference in growth was already detectable during the second trimester of pregnancy (Nelissen et al., 2013) and was present at least up to the age of 2 years (Kleijkers et al., 2014). These findings have, since then, been both supported and disputed in studies comparing other culture media (Zandstra et al., 2015).

We conducted a large multicenter, randomized, double-blind trial comparing live birth rates as well as pregnancy and perinatal outcomes after IVF using one of the two culture media that have been used worldwide in IVF programs, namely human tubal fluid (HTF) medium and G5 medium.

Materials and Methods

Trial design

In this multicenter RCT, couples were randomly assigned to have their oocytes and embryos cultured in one of the two media, i.e. G5 (Vitrolife, Göteborg, Sweden) and HTF (Lonza Verviers, Belgium). The end-of-study participation for a couple was the achievement of a live birth, the passing of 1 year after randomization or withdrawal of consent by the couple. The study was conducted at six hospital-based IVF centers in the Netherlands (Academic Medical Center in Amsterdam (AMC), Catharina Hospital in Eindhoven, St. Elisabeth Hospital in Tilburg, Maastricht University Medical Center in Maastricht (MUMC), University Medical Center Groningen in Groningen (UMCG), and Radboud university medical center in Nijmegen (UMCN)) and four of their affiliated clinics (Onze Lieve Vrouwe Gasthuis in Amsterdam, Gemini Hospital in Den Helder, Scheper Hospital in Emmen, and Maxima Medical Center in Veldhoven). The study protocol was approved by the Central Committee on Research Involving Human Subjects (CCMO) in the Netherlands and by the institutional review boards of all participating centers. The study was registered in the Netherlands Trial Registry (NTR1979).

Randomization

Randomization was performed centrally by an online computer program with a 1:1 allocation using random block sizes of two and four couples, 1 day before oocyte retrieval of the first cycle. For each individual center, stratification was performed for fertilization technique (IVF or ICSI) and maternal age (<38 and ≥38 years of age). Allocation sequence and allocated treatment were fully blinded to participating couples, attending gynecologists, fertility doctors and outcome assessors. Blinding of the embryologists was not possible since they performed the procedures in the laboratory. At the end of the study, when data collection was completed, the allocation sequence was revealed to the primary investigators.

Participants

Couples that were scheduled for an IVF or ICSI treatment for their first IVF/ICSI cycle ever or first IVF/ICSI cycle after a previous successful pregnancy were eligible to participate in the study. Couples undergoing a modified natural cycle, couples for whom IVF was used to prevent the transmission of human immunodeficiency virus, couples undergoing PGD and couples using ART for fertility preservation were excluded. All participating couples provided written informed consent for participating in the study and the use of their data.

Interventions and IVF procedures

Participating couples were randomly assigned to either the G5 culture medium group or the HTF culture medium group. The allocated medium was used in all treatment cycles, including transfers with cryopreserved embryos that the women received in the year following randomization. All other treatment procedures, such as ovarian hyperstimulation, follicular aspiration and oocyte fertilization, were performed according to the routine IVF/ICSI procedures in each particular center and were identical for both study groups.

The G5 PLUS culture medium system included G-IVF PLUS medium for fertilization, G1 PLUS medium for culturing embryos from Day 1 to Day 3 and G2 PLUS medium for culturing embryos from Day 3 to Day 4, where applicable. The G5 PLUS media were ready-to-use media already supplemented with human serum albumin. HTF medium was used for culture of oocytes and embryos from fertilization up to Day 4 of culture, where applicable. To keep procedures as similar as possible, HTF embryos were transferred to a new culture dish containing fresh HTF on Day 3 of culture. HTF was supplemented with 10% Albuman (Sanquin Plasma Products BV, Amsterdam, the Netherlands).

In case of IVF, oocytes were incubated in dishes containing 50 μ l droplets of G-IVF PLUS or HTF, according to the culture medium allocated to the couple, with 10 000–100 000 progressively motile spermatozoa for fertilization. The next morning, cumulus cells were removed and all oocytes were transferred to a clean dish containing the allocated medium, G1 PLUS or HTF. On Day 3 of culture, the embryos were transferred to a new dish containing G2 PLUS or HTF.

In case of ICSI, the oocytes were denuded using cumulus (Origio, Malov, Denmark), hyadase (Origio, Malov, Denmark) or hyase (Vitrolife, Göteborg, Sweden), injected with a single immobilized spermatozoon and directly thereafter cultured in dishes containing G1 PLUS or HTF according to the treatment allocation. On Day 3 of culture, the embryos were transferred to a new dish containing G2 PLUS or HTF.

Embryo culture was performed following the manufacturer's instructions, i.e. at 37°C and 6% CO₂ for G5 and 37°C and 5% CO₂ for HTF. Three centers cultured oocytes and embryos in 5% O₂ (AMC, MUMC and St. Elisabeth Hospital), whereas the other three cultured oocytes and embryos in air (18–20% O₂; Catharina Hospital, UMCG and UMCN). Embryo morphology was assessed daily and the number of cells and the percentage of fragmentation were scored based on a structured scoring sheet available in all centers ([Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011](#)). All embryologists in all centers participate in a national external online embryo scoring quality control scheme to limit inter-individual variation in embryo scoring (www.embryo-online.eu). Embryos of sufficient quality were transferred to the uterine cavity of the women on Day 2 (UMCG, MUMC) or 3 (AMC, Catharina Hospital, MUMC, St. Elisabeth Hospital, UMCN) after fertilization according to the local policies of each participating center. Cryopreservation of supernumerary good quality embryos was performed with a slow freeze technique according to the local protocol on Day 3 (MUMC, UMCG, UMCN), 4 (AMC, Catharina Hospital, St. Elisabeth Hospital) or 5 (St. Elisabeth Hospital) after fertilization. After thawing, the allocated medium was used for culture and transfer of these embryos. A new IVF/ICSI cycle was initiated only after all cryopreserved embryos from the previous cycle were thawed and transferred.

Outcomes

The primary outcome was live birth rate, which was defined as the proportion of couples that achieved a birth of at least one baby born alive, independent of gestational age (GA). Secondary outcomes were number of utilizable embryos, implantation rate, biochemical pregnancy, clinical pregnancy, ongoing pregnancy, miscarriage, birthweight and congenital abnormalities.

The number of utilizable embryos was defined as the number of embryos transferred plus the number of embryos cryopreserved. The number of implantations was determined by the number of fetal sacs as identified by transvaginal ultrasound examination at 6–8 weeks of gestation. A biochemical pregnancy was defined as a serum β hCG level of at least 50 IU/l 2 weeks after embryo transfer. Clinical pregnancy was determined by the presence of a gestational sac and fetal heartbeat confirmed by transvaginal ultrasound examination at 6–8 weeks of gestation. Ongoing pregnancy was defined as a viable intrauterine pregnancy after 12 weeks of gestation. A miscarriage was determined by a biochemical pregnancy not resulting in a live birth.

After delivery, questionnaires were sent to all women and their obstetrician or midwife. Questionnaires included questions about ultrasound examinations, pregnancy characteristics and complications (i.e. gestational diabetes, hypertension and pre-eclampsia) and perinatal outcome. Congenital malformations were classified as major when they caused functional impairment or required surgical correction. The remaining congenital malformations were considered minor ([Bonduelle et al., 2002](#)). Actual GA was calculated based on the day of oocyte retrieval, which was defined as Day 14 of the cycle. Birthweight percentiles were based on the median weight of a reference population of children born at the same GA and of the same gender ([Oken et al., 2003](#)). These were used to define very small for GA (<3rd percentile), small for GA (<10th percentile), large for GA (>90th percentile) and very large for GA (>97th percentile) infants and to calculate z-scores. In case of a twin pregnancy, the inter-twin birthweight disparity was calculated in grams and the inter-twin birthweight discordance as a percentage by dividing the difference in birthweight by the weight of the heavier twin and multiplying the result by 100% ([Blickstein and Kalish, 2003](#)).

To investigate fetal growth, data from the ultrasound pregnancy examinations were used. In the Netherlands, all pregnant women are offered ultrasound dating during the first trimester and are counseled for fetal ultrasound examination around 20 weeks of gestation to have fetal biometry performed and be screened for structural abnormalities. Furthermore, women can freely choose to participate in first-trimester screening for Down syndrome. To compare fetal growth between the study groups, while adjusting for the differences in GA at which the examinations were performed, differences between the estimated GA (based on data from the ultrasound examinations) and actual GA (oocyte retrieval based) were used (Δ GA) and expressed in days. The crown-rump-length (CRL)-based formula by [Hadlock et al. \(1992\)](#) was used to calculate the GA at 8 and 12 weeks of gestation and the biparietal diameter (BPD)-based formulae by [Mul et al. \(1996\)](#) #1 and #2 and [Selbing and Kjessler \(1985\)](#) were used to calculate the GA at 20 weeks of gestation ([Saltvedt et al., 2004](#)). Furthermore, fetal weight was estimated based on several sonographic parameters (head circumference, abdominal circumference, femur length and BPD) that were measured during the mid-trimester ultrasound scan at 20 weeks of gestation using the [Hadlock et al. \(1985\)](#) I, III and IV formulae ([Hoopmann et al., 2010](#)).

Sample size and interim analysis

Based on a live birth rate of 45% after 1 year of IVF/ICSI treatment in the participating centers in the years preceding this study, we calculated that at least 784 women would be needed to detect a difference of 10% in live birth rate after 1 year of treatment with a power of 80% at an α of 0.05. More patients were counseled and invited to participate in the study to allow for dropouts.

One interim analysis of efficacy was planned to be performed 1 year after the initiation of the study by an independent data and safety monitoring committee. Primary outcome of this interim analysis was ongoing pregnancy for first, second and third cycles, including transfers with frozen–thawed embryos, that had been performed at that time. The data and safety

monitoring committee had to use these proxy outcomes as otherwise an interim analysis would not have been possible before the completion of the inclusion of the study. Live birth data were available only at the end of the study, due to the design and inclusion rate of the study. For the same reason, no formal stopping rules or adjusted statistics were applied. A blinded overview, which was available only to the data and safety monitoring committee, was used for the interim analysis.

Statistical analysis

We calculated rates of pregnancy and live birth in each group and the corresponding rate ratios (RRs) with 95% confidence intervals (95% CI). Differences in outcomes were statistically evaluated using χ^2 statistics for categorical variables and one-way analysis of variance for continuous variables. Data were analyzed according to the intention-to-treat principle. Birthweight differences between children from the G5 group versus the HTF group were analyzed by means of general estimating equations (Zeger and Liang, 1986), in view of the statistical dependence between birthweights within twin pairs. In addition, being part of a twin was added to the analysis as a covariable because of its strong association with birthweight. Perinatal outcome was further investigated in singletons and twins separately, as beforehand twinning was considered to be an important confounder and a treatment-related bias as the study was not designed to include single embryo transfers only. To investigate the effect of medium on birthweight controlled for other potential confounders, a multivariable regression analysis was performed and regression imputation was used for missing data on parental height, weight and smoking.

Results

Between July 2010 and May 2012, a total of 836 couples were randomly allocated to undergo IVF/ICSI using either G5 ($n = 417$) or HTF ($n = 419$) medium for embryo culture (Fig. 1). Four hundred and twelve couples in the G5 group and 395 couples in the HTF group underwent the allocated intervention in all their cycles. Five couples in the G5 and 24 couples in the HTF group did not receive the allocated treatment in one of the performed cycles because of human error in identifying the couple as participating in the study; in these couples, the medium used for non-trial participants in that specific center was used. Only one couple (in the G5 group) withdrew consent prior to finishing the year of treatment, after two cycles. The couple had not achieved pregnancy and was analyzed as such. Nineteen couples were allocated in either of the two culture media without fulfilling the inclusion criteria, the primary reason being that they had undergone one or more previous unsuccessful IVF/ICSI treatments. Since our analysis is based on intention-to-treat, those couples were included in the analysis.

The couples remained enrolled in the study for 1 year after allocation and in case of an ongoing pregnancy they were followed until delivery. Only one of the couples with an ongoing pregnancy (in the G5 group) was lost to follow up. We included this pregnancy as not resulting in a live birth in our analysis. The baseline characteristics of the couples are presented in Table 1. Four couples (3 in the G5 group and 1 in the HTF group) received oocyte donation and 22 couples (8 in the G5 group

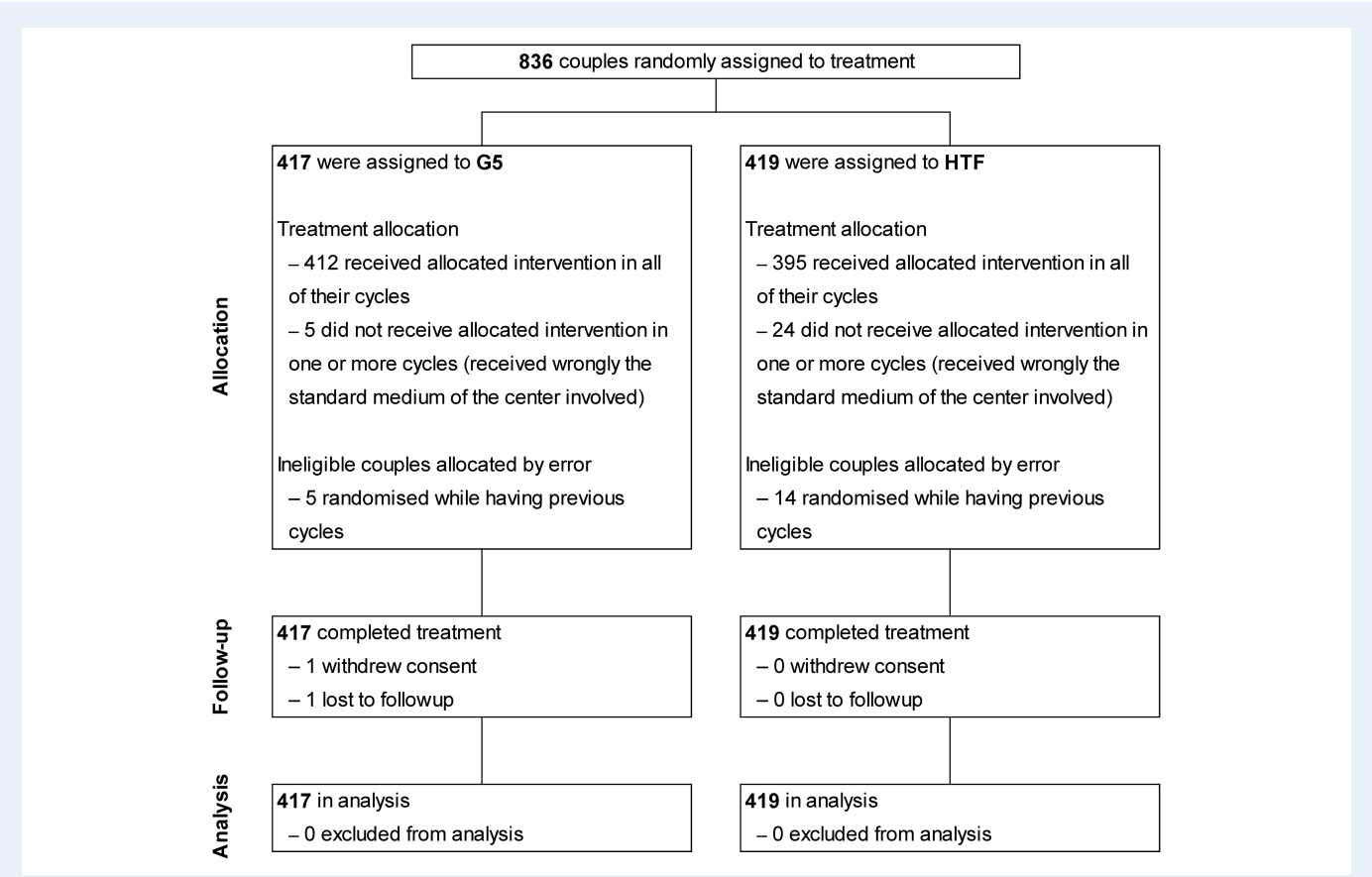


Figure 1 Flow chart of allocation, follow-up and analysis of the included couples in an RCT of the influence of embryo culture medium (G5 and HTF) on pregnancy and perinatal outcome after IVF.

Table 1 Baseline characteristics of the couples in an RCT of the influence of embryo culture medium (G5 and HTF) on pregnancy and perinatal outcome after IVF.

Characteristic	All couples		Couples with a live born child	
	G5 (n = 417)	HTF (n = 419)	G5 (n = 184)	HTF (n = 159)
Primary indication for IVF/ICSI				
Tubal	47 (11.3)	37 (8.8)	20 (10.9)	12 (7.5)
Male subfertility	211 (50.6)	225 (53.7)	104 (56.5)	94 (59.1)
Unexplained	100 (24.0)	84 (20.0)	34 (18.5)	28 (17.6)
Other	59 (14.1)	73 (17.4)	26 (14.1)	25 (15.7)
Maternal characteristics				
Age (years)	33.9 ± 4.3	33.8 ± 4.4	32.9 ± 3.9	33.0 ± 4.1
Height (cm) ^a	n/a	n/a	169.8 ± 7.6	171.0 ± 7.0
Weight (kg) ^a	n/a	n/a	68.6 ± 12.6	71.1 ± 12.7
Smoking ≥ 10 cigarettes/day ^a	n/a	n/a	13 (7.1)	16 (10.1)
Paternal characteristics				
Age (years)	37.5 ± 6.2	37.3 ± 6.4	36.7 ± 6.2	37.0 ± 6.4
Height (cm) ^a	n/a	n/a	183.1 ± 7.5	182.9 ± 7.6
Weight (kg) ^a	n/a	n/a	87.7 ± 13.6	88.1 ± 13.3
Smoking ≥ 10 cigarettes/day ^a	n/a	n/a	26 (14.1)	21 (13.2)
Duration of subfertility (years)	3.1 ± 1.9	3.1 ± 2.3	3.1 ± 1.8	3.3 ± 2.5
Nulliparous	312 (74.8)	317 (75.7)	133 (72.3)	115 (72.3)

Data presented as mean ± SD or numbers (%). n/a denotes not available.

^aData on maternal height, maternal weight, maternal smoking, paternal height, paternal weight and paternal smoking were missing from 26 (7.6%), 28 (8.2%), 28 (8.2%), 27 (7.9%), 32 (9.3%) and 38 (11.1%) couples, respectively.

and 14 in the HTF group) had treatment with sperm from a donor. In these cases, the age of the donor was used for the calculation of the mean maternal and paternal age. No important differences were found between baseline characteristics of the G5 group and the HTF group, either among all couples or in the subgroup of couples with a live born child (Table 1).

IVF outcomes

After 1 year of treatment, live birth rate was not significantly different for women in the G5 group when compared with women in the HTF group (44.1% (184/417) versus 37.9% (159/419); RR: 1.2; 95% CI: 0.99–1.37; $P = 0.08$; mean difference 6.2%, 95% CI: –0.5 to 12.9). Similarly, there was no difference in the percentage of women achieving an ongoing pregnancy between the G5 group and the HTF group (45.3% (189 of 417) versus 38.7% (162 of 419); RR: 1.2; 95% CI: 0.99–1.37; $P = 0.06$). Women in the G5 group had a significantly higher rate of clinical pregnancy than women in the HTF group (47.7% (199 of 417) versus 40.1% (168 of 419); RR: 1.2; 95% CI: 1.02–1.39; $P = 0.03$), whereas the proportion of miscarriages did not differ between the study groups (Table II).

A total of 1497 cycles (713 (mean of 1.71) for the G5 group and 784 (mean of 1.87) for the HTF group) were performed (1.71 ± 0.04 versus 1.87 ± 0.04 ; $P = 0.003$). There was no difference in the percentage of cycles with ICSI (58.3% (416 of 713) versus 57.3% (449 of 784); RR: 1.0; 95% CI: 0.93–1.11; $P = 0.68$) or the proportion of oocyte retrievals resulting in a fresh transfer (93.0% (663 of 713) versus 93.5% (733 of 784); RR: 1.0; 95% CI: 0.97–1.02; $P = 0.76$) between the G5 group

and the HTF group (Supplementary Table SI). The percentage of transfers using cryopreserved embryos in the G5 group was higher than in the HTF group (38.7% (276 of 713) versus 26.0% (204 of 784); RR: 1.5; 95% CI: 1.28–1.73; $P < 0.001$) (Supplementary Table SI).

The embryo characteristics of all treatments are illustrated in Table III. Fertilization rate was significantly lower in the G5 group than in the HTF group (62.9% (3655 of 5807) versus 69.1% (4341 of 6286); $P < 0.001$). The mean number of utilizable embryos per cycle, i.e. the mean number of embryos that were transferred or cryopreserved per cycle, was significantly higher in the G5 group than in the HTF group (2.8 ± 2.3 versus 2.3 ± 1.8 ; $P < 0.001$). Furthermore, the proportion of embryos that implanted after fresh transfer was significantly higher in the G5 group than in the HTF group (20.2% (195 of 966) versus 15.3% (171 of 1117); $P < 0.001$). At the end of the study period, significantly more embryos per woman were still cryopreserved in the G5 group when compared with the HTF group (mean 1.28 ± 0.12 versus 0.79 ± 0.09 ; $P = 0.001$).

Perinatal outcome

Of the 343 pregnancies with live births, 301 were single gestation pregnancies (G5: 164, HTF: 137), whereas 42 were multiple gestation pregnancies (G5: 20, HTF: 22). In two multiple gestation pregnancies with live births (G5: 1, HTF: 1), one of the fetuses was lost between 12 weeks of gestation (ongoing pregnancy) and birth. From this, a total of 383 live born children were included in the study, 165 singleton and 38 (18.7%) twin children in the G5 group and 138 singleton and 42 (23.3%) twin children in the HTF group.

Table II Outcomes in women allocated to G5 and HTF.

Outcome	G5 (n = 417)	HTF (n = 419)	Risk ratio (95% CI) ^a	P-Value
Women with ≥ 1 child born (live birth rate)	184 (44.1)	159 (37.9)	1.2 (0.99–1.37)	0.08
Total no. of live births ^b	203	180		
Women with ≥ 1 ongoing pregnancy ^c	189 (45.3)	162 (38.7)	1.2 (0.99–1.37)	0.06
Total no. of ongoing pregnancies	190	163		
Women with ≥ 1 clinical pregnancy	199 (47.7)	168 (40.1)	1.2 (1.02–1.39)	0.03
Total number of clinical pregnancies	205	170		
Women with ≥ 1 biochemical pregnancy	236 (56.6)	210 (50.1)	1.1 (0.99–1.28)	0.06
Total number of biochemical pregnancies	255	221		
Women with ≥ 1 miscarriage	66 (15.8)	56 (13.4)	1.2 (0.85–1.65)	0.33
Total number of miscarriages	71	62		

CI, confidence interval.

Data presented as number (%).

^aRisk ratio for G5 group when compared with HTF group.^b165 singleton and 38 twin children in the G5 group; 138 singleton and 42 twin children in the HTF group.^cTwo pregnancies with stillborn, two pregnancies with intrauterine deaths and one pregnancy loss to follow-up in the G5 group; one pregnancy with stillborn (twin) and two pregnancies with intrauterine deaths in the HTF group.**Table III Embryologic characteristics according to the treatment.**

Characteristic	G5	HTF	P-Value
Cumulus–oocyte complexes: number (mean number per cycle \pm SD)	6461 (9.1 \pm 5.3)	7102 (9.1 \pm 5.4)	0.99
Oocytes inseminated/injected: number (mean number per cycle \pm SD)	5807 (8.1 \pm 5.0)	6286 (8.0 \pm 5.0)	0.62
Fertilized oocytes (embryos) ^a : number (mean number per cycle \pm SD)	3655 (5.1 \pm 3.9)	4341 (5.5 \pm 4.0)	0.04
Fertilization rate: % per oocytes inseminated/injected	62.9	69.1	<0.001
Utilizable embryos ^b : number (mean number per cycle \pm SD)	1966 (2.8 \pm 2.3)	1779 (2.3 \pm 1.8)	<0.001
Embryos transferred in a fresh cycle: number (mean number per transfer \pm SD)	966 (1.5 \pm 0.6)	1117 (1.5 \pm 0.6)	0.19
Implanted embryos from a fresh cycle: number (% of embryos transferred)	195 (20.2)	171 (15.3)	<0.001
Embryos cryopreserved: number (mean number per cycle \pm SD)	1000 (1.4 \pm 2.4)	662 (0.8 \pm 1.8)	<0.001
Cryopreserved embryos transferred: number (mean number per transfer with cryopreserved embryos \pm SD)	325 (1.2 \pm 0.6)	225 (1.1 \pm 0.6)	0.20
Implanted cryopreserved embryos: number (% of cryopreserved embryos transferred)	35 (10.7)	25 (11.1)	0.89

The differences in outcomes between study groups were tested by one-way analysis of variance.

^a2PN zygotes and 1PN/0PN zygotes that cleaved in 48 h.^bNumber of embryos transferred in a fresh cycle or cryopreserved.

Of the 383 live born children, birthweight data from 380 children (300 singletons (G5: 163, HTF: 137) and 80 twin children (G5: 38, HTF: 42)) were retrieved. Birthweight was significantly lower in the G5 group compared with the HTF group, with a mean difference of 158 g (95% CI: 42–275 g; $P = 0.008$). Separate analysis for singletons and twins is provided in Tables IV and V, respectively. Birthweight of the singletons was also significantly lower in the G5 group compared with the HTF group (3299 ± 46 g versus 3480 ± 44 g; $P = 0.005$), whereas there was no difference in birthweight for twins from the G5 or HTF group. Furthermore, singleton birthweight adjusted for GA and gender (z-score) was lower in the G5 group than in the HTF group (-0.13 ± 0.08 versus 0.17 ± 0.08 ; $P = 0.008$). In the G5 group, more singletons were born preterm (8.6% (14/163) versus 2.2% (3/137); RR: 3.92; 95% CI: 1.15–13.37; $P = 0.02$) and there

were more singletons with low birthweight (<2500 g) (9.8% (16/163) versus 2.9% (4/137); RR: 3.36; 95% CI: 1.15–9.82; $P = 0.02$). The percentage of children with major or minor malformations did not differ between the groups (Tables IV and V).

To determine how the effect of culture medium on birthweight relates to the effect of other possible birthweight confounders, a multivariate analysis was performed. Multiple linear regression indicated that the adjusted mean birthweight of singletons was 116 g lower in the G5 group than in the HTF group (95% CI: -212 to -20 ; $P = 0.02$) (Supplementary Table SII). Additionally, in the subgroup of 283 singletons that were born from term pregnancies (≥ 37 weeks), the adjusted mean birthweight of singletons in the G5 group was 100 g lower than in the HTF group (95% CI: -198 to -2 ; $P = 0.04$). It has been shown that the duration of culture can affect birthweight in humans (Makinen

Table IV Neonatal outcome of live born singletons.

Outcome	G5 (n = 163)	HTF (n = 137)	Risk ratio (95% CI) ^a	P-Value
Female	71 (43.6)	65 (47.4)	0.92 (0.72–1.18)	0.56
Birthweight (g)	3299 ± 46	3480 ± 44		0.005
z-Score	−0.13 ± 0.08	0.17 ± 0.08		0.008
GA at birth (weeks)	39.2 ± 0.1	39.4 ± 0.1		0.19
Preterm birth (<37 weeks)	14 (8.6)	3 (2.2)	3.92 (1.15–13.37)	0.02
Very preterm birth (<32 weeks)	1 (0.6)	0 (0.0)	n/a	1.00
Low birthweight (<2500 g)	16 (9.8)	4 (2.9)	3.36 (1.15–9.82)	0.02
Low birthweight with GA ≥ 37 weeks	6 (3.7)	3 (2.2)	1.68 (0.43–6.60)	0.52
Very low birthweight (<1500 g)	1 (0.6)	0 (0.0)	n/a	1.00
High birthweight (>4500 g)	1 (0.6)	4 (2.9)	0.21 (0.02–1.86)	0.18
Small for GA (<10th percentile)	14 (8.6)	5 (3.6)	2.35 (0.87–6.37)	0.10
Very small for GA (<3rd percentile)	4 (2.5)	1 (0.7)	3.36 (0.38–29.73)	0.38
Large for GA (>90th percentile)	15 (9.2)	16 (11.7)	0.79 (0.41–1.54)	0.57
Very large for GA (>97th percentile)	2 (1.2)	7 (5.1)	0.24 (0.05–1.14)	0.09
Major malformations	4 (2.5)	6 (4.4)	0.56 (0.16–1.95)	0.52
Minor malformations	6 (3.7)	6 (4.4)	0.84 (0.28–2.55)	0.78

GA, gestational age; n/a, not applicable

Data presented as mean ± SEM or numbers (%). The differences in outcomes between study groups were tested by one-way analysis of variance for continuous variables and χ^2 test for categorical variables.^aRisk ratio for G5 group when compared with HTF group.**Table V Neonatal outcome of live born twins.**

Outcome	G5 (n = 38)	HTF (n = 42)	Risk ratio (95% CI) ^a	P-Value
Female	23 (60.5)	17 (40.5)	1.50 (0.96–2.34)	0.12
GA at birth (weeks)	35.6 ± 0.4	35.1 ± 0.4		0.47
Preterm birth (<37 weeks)	26 (68.4)	30 (71.4)	0.96 (0.72–1.28)	0.81
Very preterm birth (<32 weeks)	4 (10.5)	8 (19.0)	0.55 (0.18–1.69)	0.36
Low birthweight (<2500 g)	21 (55.3)	28 (66.7)	0.83 (0.58–1.19)	0.36
Low birthweight with GA ≥ 37 weeks	4 (10.5)	4 (9.5)	1.11 (0.30–4.12)	1.00
Very low birthweight (<1500 g)	6 (15.8)	5 (11.9)	1.33 (0.44–4.00)	0.75
Major malformations	1 (2.6)	2 (4.8)	0.55 (0.05–5.85)	1.00
Minor malformations	1 (2.6)	0 (0.0)	n/a	0.48
Birthweight (g)	2266 ± 100	2267 ± 94		0.99
Birthweight disparity (g)	426 ± 64	289 ± 40		0.07
Birthweight discordance (%)	17.1 ± 2.4	12.4 ± 1.7		0.11
Birthweight discordance (≥25%)	10 (26.3)	8 (19.0)	1.38 (0.61–3.14)	0.59

n/a, not applicable.

Data presented as means ± SEM or numbers (%).

^aRisk ratio for G5 group when compared with HTF group.

et al., 2013; Zhu et al., 2014b; Kaartinen et al., 2015). Furthermore, it has been suggested that oxygen concentration during culture can influence the effect of culture medium, at least on blastocyst formation in the mouse (Morbeck et al., 2014a). As those factors are related to center of treatment in this study, an additional analysis including center of treatment was performed. It was found that embryo culture medium

(G5 versus HTF) was still associated with singleton birthweight ($\beta = -117$ g; 95% CI: -213 to -20 ; $P = 0.02$).

Fetal growth

To investigate the onset of the intrauterine growth difference between the children from the G5 group and the HTF group, the fetal growth

Table VI Fetal growth in single gestation pregnancies with a live birth.

Outcome	G5	HTF	P-Value
8 weeks of gestation (n)	149	129	
ΔGA; Hadlock et al. (1992)	-0.4 ± 0.1	-0.5 ± 0.2	0.762
12 weeks of gestation (n)	60	54	
ΔGA; Hadlock et al. (1992)	2.3 ± 0.7	3.6 ± 0.9	0.244
20 weeks of gestation (n)	139	110	
ΔGA; Mul et al. (1996) (#1)	1.2 ± 0.4	0.7 ± 0.4	0.360
ΔGA; Mul et al. (1996) (#2)	0.8 ± 0.4	0.3 ± 0.4	0.342
ΔGA; Selbing and Kjessler (1985)	3.7 ± 0.4	3.1 ± 0.5	0.421
20 weeks of gestation (n)	138	111	
EFW; Hadlock I (g)	335 ± 4	343 ± 5	0.199
EFW; Hadlock III (g)	341 ± 4	349 ± 5	0.230
EFW; Hadlock IV (g)	331 ± 4	340 ± 5	0.197

EFW, estimated fetal weight.

Data are presented as mean \pm SEM.

patterns in single gestation pregnancies were compared between the study groups. Around 8 weeks of gestation, fetal CRL was retrieved in 149 (90.9%) single gestation pregnancies from the G5 group and 129 (94.2%) single gestation pregnancies from the HTF group. Not all women participated in the Down screening around 12 weeks of gestation, and fetal CRL was retrieved in only 114 single gestation pregnancies, 60 (36.6%) in the G5 group and 54 (39.4%) in the HTF group. In the screening for structural abnormalities of the fetus around 20 weeks of gestation, not all biometric parameters used to calculate the ΔGA or estimated fetal weight (EFW) were measured in all women. For ΔGA around 20 weeks of gestation, data from 139 (84.8%) pregnancies from the G5 group and 110 (80.3%) pregnancies from the HTF group were analyzed, whereas for EFW, data from 138 (84.1%) G5 pregnancies and 111 (81.0%) HTF pregnancies were analyzed. Intrauterine growth of singletons from single gestation pregnancies is presented in Table VI. The differences between the CRL- or BPD-based GA and actual (oocyte retrieval based) GA were not different between the study groups at 8, 12 and 20 weeks of gestation. Furthermore, the EFW at 20 weeks of gestation, as calculated with three different formulae by Hadlock, did not differ between the two study groups.

Discussion

In this randomized double-blinded trial, we evaluated the effect of two embryo culture media that are used in IVF/ICSI treatments and found a 6% difference in live births in favor of G5 medium. Despite the absence of a statistical significance in the difference in live birth rate (G5 versus HTF; RR: 1.2; 95% CI: 0.99–1.37), we conclude that G5 provides better treatment success than HTF, as other outcomes that are relevant for IVF and correlate to live birth rate, all significantly favored G5. This includes clinical pregnancy, number of utilizable embryos, number of embryos implanted after fresh transfer and the number of cryopreserved embryos that remained available at the end of the study. The beneficial effect of G5 on all these outcomes but live birth

rate is probably due to the study being powered to detect a 10% difference in live births. Future designs should include a larger number of patients to be able to establish smaller differences, which are still clinically relevant.

Birthweight was significantly lower in children from the G5 group compared with children from the HTF group, with a mean difference of 158 g (95% CI: 42–275 g; $P = 0.008$). In previous studies with consecutive or alternate use of different culture media, some reported effects of culture medium on birthweight (Dumoulin et al., 2010; Nelissen et al., 2012; Eskild et al., 2013; Hassani et al., 2013; Zhu et al., 2014a,b), whereas others found no effect (Eaton et al., 2012; Vergouw et al., 2012; Carrasco et al., 2013; Lin et al., 2013; Ziebe et al., 2013; Lemmen et al., 2014; Wunder et al., 2014) [see review by Zandstra et al. (2015)]. We now, for the first time by means of a large RCT, confirm that embryo culture medium can have an effect on birthweight in humans. The clinical relevance of a difference in birthweight after IVF, the mechanism through which a few days of embryo culture affects birthweight 9 months later, and a more optimal composition of culture media for IVF must now be determined.

There were more preterm births, with lower birthweight, among singletons in the G5 group. This does not fully explain; however, the observed difference in birthweight as well as the z-score (birthweight adjusted for GA and gender) was significantly lower in the G5 group than in the HTF group. It is well known that children born after ART have a higher risk for preterm birth and low birthweight compared with children born after natural conception (Henningsen and Pinborg, 2014). Our study suggests embryo culture medium composition is one of the causative factors for this.

The fetal growth patterns from both study groups did not differ up to 20 weeks of gestation, which indicates that the onset of the growth differentiation is distinguishable only after this period. This is not in agreement with a study by Nelissen et al. (2013), in which it was found that the fetal growth patterns diverged already at 20 weeks of gestation after the use of two different culture media. It has been suggested that many ART procedures, including certain culture conditions, are associated with fetal growth restriction in early to mid-pregnancy, succeeded by substantial increases in placental size and accelerated fetal growth toward the end of gestation (Bloise et al., 2014). In mice, it was shown that culture medium composition highly determines fetal weight after ART (Delle Piane et al., 2010).

Although speculative, the effects that embryo culture media have on birthweight of newborns could be mediated through a direct effect on the epigenome of the developing preimplantation embryo (van Montfoort et al., 2012). In recent publications, we demonstrated that the use of G5 and HTF resulted in different gene expression profiles in human preimplantation embryos at the blastocyst stage (Kleijkers et al., 2015; Mantikou et al., 2016). A side-by-side comparison of five human culture media indicated that all had a varying but compromised ability to maintain gene expression and DNA methylation patterns in mouse preimplantation embryos when compared with *in vivo*-derived embryo (Market-Velker et al., 2010). Alternatively, it could be that embryo culture media affect the ability of preimplantation embryos to properly implant. In such a scenario, the observed lower birthweight, as well as the increased incidence of preterm birth, would be an indirect effect of culture media via disturbed implantation.

It is too early to know whether a difference of 158 g in birthweight has any clinical significance. However, an inverse association between

birthweight and increased rates of coronary heart disease and the related disorders of stroke, hypertension and Type 2 diabetes during adult life has previously been demonstrated (Barker, 1997). This has led to the Barker or developmental origin of health and disease (DOHaD) hypothesis, which suggests that the fetus is sensitive to its (uterine) environment, and that adaptations of the fetus to its environment have lasting consequences for its development, growth and health. A well-known example of the DOHaD hypothesis is the finding that individuals conceived during the Dutch famine of the World War II have higher rates of coronary heart disease, a more atherogenic lipid profile, disturbed blood coagulation, increased stress responsiveness and are more often obese than unexposed individuals or those exposed during mid- or late-gestation, even without an effect on birthweight (Roseboom *et al.*, 2011). It has also been suggested that preimplantation embryos or even gametes adapt to their altering environment in response to parental diet with long-term consequences for health of the future adult (Carone *et al.*, 2010; Fleming *et al.*, 2012; Lucas, 2013).

One of the differences between the embryo culture media is that the G5 medium contains certain amino acids and that the HTF medium completely lacks amino acids. Studies in human and animal embryos have shown that the embryo development *in vitro* can indeed be improved by the addition of amino acids to the culture medium (Liu and Foote, 1995; Devreker *et al.*, 1998; Lane and Gardner, 1998; Summers and Biggers, 2003). The importance of amino acids during preimplantation embryo development also stems from studies in which mice were given a low protein diet (LPD) only during the period of conception. The offspring had a higher birthweight and blood pressure, even when the embryos were transferred to a pseudo-pregnant mouse that did not have the LPD during conception (Watkins *et al.*, 2008; Fleming *et al.*, 2012). We are not aware of any study that has looked specifically at the effect of amino acids in embryo culture media on pregnancy rates, live births or perinatal outcome in humans. Other notable differences in composition are the addition of hyaluronan and lipoic acid to G5. A meta-analysis showed that the use of hyaluronan-enriched transfer media resulted in increased implantation and live birth rates (Bontekoe *et al.*, 2014). Although lipoic acid is thought to improve mouse embryo development via its role as antioxidant (Talebi *et al.*, 2012), no studies have evaluated its effect on pregnancy or live birth rates in humans and no studies have looked at the effect of hyaluronan- or lipoic acid enrichment of embryo culture media on perinatal outcome. The protein source in the culture media is also an important candidate in this respect, as the quality of it has been shown to vary considerably between batches as well as between manufacturers (Dyrlund *et al.*, 2014; Morbeck *et al.*, 2014b) and the protein source has previously been suggested to influence live birth rate (Meintjes *et al.*, 2009) and birthweight (Zhu *et al.*, 2014a).

It is relevant to note that HTF was widely used for many years in IVF worldwide, but has lost significant market share in recent years. Embryologists now seem to prefer other, more 'enriched media', i.e. media that contain additives such as the previously mentioned amino acids. Still, HTF is not completely removed from the market. At the time of study design, 8 out of the 13 IVF centers in the Netherlands used HTF medium to culture preimplantation embryos. Based on the Dutch national reports of the IVF results (NVOG), there were no clear differences in ongoing pregnancy rates between centers using HTF medium and the other centers. Two small studies published as abstracts indicated that G2 medium (G2 is an earlier version of G5 and is no longer on the market) had a higher efficacy than HTF medium (Bisioli *et al.*, 2003;

Choi *et al.*, 2004); however, well-designed RCTs comparing these media to more classic media were lacking. Proper trials to support these assumptions are very much needed according to the good clinical practice guidelines (Mantikou *et al.*, 2013).

Over 20 other embryo culture media are commercially available. A trial comparing all available culture media simultaneously is simply not possible. Nevertheless, such an essential component of IVF should be treated with the highest level of scrutiny, and there is a need for more well-designed RCTs on media for human embryo culture focusing not only on pregnancy rates but also on birthweight and child health. In addition, the full composition of embryo culture media should be made publicly available by the companies that produce them (Summers and Biggers, 2003). Recently, a composition analysis of certain components was performed for some embryo culture media, but not all (Morbeck *et al.*, 2014a). The formulations should have a scientific rationale and the introduction of new media into clinical practice should be based on properly conducted RCTs. Companies should also report what studies have been performed to test these media and which end-points have been analyzed. In our view, the responsibility for proper introduction of embryo culture media with new formulations lies not only with the manufacturers, but also with clinical embryologists that decide to implement these culture media in clinical practice. The legislative background behind IVF culture media is such that there are limited constraints on introducing new culture media, even if they contain new growth factors of unknown effect on pregnancy rates, let alone the health of children born. We feel the time has come to radically shift gears in our field and that we should no longer blindly accept new culture media (or other alterations in laboratory or clinical procedures) without first rigorously studying effectiveness and safety, not unlike the introduction of new drugs or medical devices.

Ongoing pregnancy or live birth rate is generally considered to be the most important outcome after IVF (Land and Evers, 2003; Braakhkke *et al.*, 2014). Our study should raise awareness of the fact that perinatal outcomes are just as important and that follow-up procedures should be an integral part of the treatment protocol in every IVF center worldwide. It has been well argued that professionals in the field of reproduction have the moral obligation to set up effectiveness and safety studies, and that the liability in this cannot simply be left to the patient by means of 'informed consent' (Dondorp and de Wert, 2011). While a patient could in principle agree to being treated with a technique of unknown safety and effectiveness, the clinician still remains responsible for what he or she does (Dondorp and de Wert, 2011).

In conclusion, we have demonstrated a significant effect of embryo culture media used in IVF on pregnancy outcome and on the birthweight of newborns. This suggests that the millions of human embryos that are cultured *in vitro* each year are sensitive to their environment. These findings should lead to increased awareness, mechanistic studies and legislative adaptations to protect IVF offspring during the first few days of their existence.

Supplementary data

Supplementary data are available at <http://humrep.oxfordjournals.org/>.

Authors' roles

J.C.M.D. and S.M. initiated and designed the trial with input from the other authors. E.M. and S.H.M.K. coordinated data collection and

quality control of data with support, input and oversight from A.M.W., A.P.A.v.M., D.C., E.S., J.C.M.D., J.v.E.-A., S.M. and S.R. Data analysis was performed by E.M., L.J.M.S., M.v.W. and S.H.M.K., which was interpreted by all authors. E.M. and S.H.M.K. drafted the report with input from all other authors. The final report has been approved by all authors.

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Conflict of interest

None declared.

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